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SEQUENCING BATCH REACTOR BIODEGRADATION OF HYDROLYZED GB AS SOLE CARBON SOURCE

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The chemical nerve agent sarin (*o*-isopropyl methylphosphonofluoridate) was hydrolyzed at 7.2 wt % in aqueous sodium hydroxide yielding primarily *o*-isopropyl methylphosphonic acid (IMPA). This hydrolysate was diluted and fed as sole carbon source to activated sludge in an aerobic sequencing batch reactor. Feed concentrations ranged from 0.25% (initial) to 0.5% (final). The reactor was operated essentially on a 15-day hydraulic residence time. Overall chemical oxygen demand removal efficiency was 86.2%, and the IMPA in the feed was quantitatively converted to methylphosphonic acid (MPA). MICROTOX® and *Daphnia magna* aquatic toxicity testing showed the effluent to be of very low toxicity to aquatic test organisms. The final MPA product was effectively absorbed by Phoslock™, which is a modified clay.

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PREFACE

The work described in this report was authorized under Project No. 778117, Assembled Chemical Weapons Assessment Program. This work was started in October 2000 and completed in September 2001.

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SEQUENCING BATCH REACTOR BIODEGRADATION OF HYDROLYZED GB AS SOLE CARBON SOURCE

1. INTRODUCTION

In 1985, the U.S. Congress directed the Department of Defense to destroy at least 90% of the unitary chemical agent stockpile that includes sarin (Public Law 99-145). This program was subsequently expanded to include the entire U.S. unitary chemical stockpile. In 1988, the Army, as documented in its Final Programmatic Environmental Impact Statement,[1] decided against the transportation of the existing untreated stockpiles to one or more central facilities and recommended destruction of the stockpiles to be accomplished at each stockpile site. By way of the National Defense Authorization Act for Fiscal Year 1993, the deadline for completion was set at December 31, 2004. Subsequently, the Chemical Weapons Convention (CWC) was signed and its entry-into-force date was April 29, 1997. The CWC requires destruction of existing stockpiles 10 years from entry-into-force. Therefore, by treaty agreement, the U.S. chemical stockpile must be destroyed by April 29, 2007.

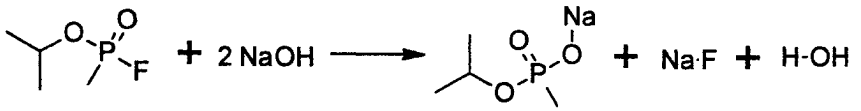
There has been an ongoing debate over the technical issues of chemical demilitarization. In 1982, the U.S. Army selected incineration as the preferred technology for destruction of the chemical stockpile. However, in response to public concerns about the use of incineration for chemical weapons disposal, the Army, under Public Law 102-484, was directed by Congress to report on alternative technologies for the disposal of chemical weapons stockpiles. Neutralization followed by biodegradation was one approach that the National Research Council recommended for further research and development.[2]

The U.S. chemical stockpile consists primarily of four chemical agents: HD (sulfur mustard, 2,2'-dichlorodiethyl sulfide), HT [consisting of 60 wt % HD and 40 wt % bis-(2-(2-chloroethylthio)ethyl) ether], VX [O-ethyl-S-2-(diisopropylaminoethyl) methyl phosphonothioate], and sarin (*o*-isopropyl methylphosphonofluoridate). The HD and HT are vesicant agents whereas VX and sarin are acetylcholinesterase inhibiting chemical nerve agents.

The hydrolysis and biodegradation of HD and HT as sole carbon source has been reported previously.[3-5] Zhang et al.[6] have demonstrated the biodegradation of sarin as sole phosphorus source in shake-flask studies. Biodegradation of sarin was also demonstrated as sole phosphorus source amended with isopropanol or glucose as the primary carbon sources[7] and in a mixed culture bioreactor amended with HEPES buffer at a final carbon to phosphorus ratio between 5:1 and 8:1 [calculated as follows: 25-50 mM HEPES yielding 2.4-4.8 g/L carbon, plus 23 mM *o*-isopropyl methylphosphonic acid (IMPA)] yielding 1.1 g/L carbon and 0.7 g/L phosphorus, for a

total carbon concentration of 3.5-5.9 g/L and a total phosphorus concentration of 0.7 g/L).[8] Final methylphosphonic acid (MPA) mineralization was accomplished as sole phosphorus source by the addition of glucose or corn syrup solids following dilution.

In the case of sarin, hydrolysis in aqueous sodium hydroxide yields the sodium salt of IMPA, NaF and water (Equation 1).



Sarin sodium salt of IMPA (1)

Analysis of reaction products typically reveals other minor products such as MPA, presumably a hydrolysis product of manufacturing impurities such as methylphosphonofluoridate. Nevertheless, IMPA is the major sarin hydrolysis product. Thus, its degradation is the central issue in the successful operation of a sarin hydrolysis product bioreactor.

Efficient sole carbon source utilization is important in biological waste treatment because biological carbon demand greatly exceeds that of biological phosphorus demand on a weight basis. Schaezler[9] conducted several studies of carbon versus phosphorus utilization by mixed cultures and pure cultures of *Escherichia coli*. His conclusion was that the stoichiometric limiting ratio of phosphorus for mixed cultures grown on glucose was between 5.4 and 6.1 mg of phosphorus per gram of glucose. Because glucose is 40% carbon by weight, his results correspond to a C:P demand ratio between 74:1 and 66:1. IMPA is 34.8% carbon and 22.4% phosphorus, so it is apparent that its efficient utilization as sole carbon source obviates the need for large amounts of exogenous carbon sources and permits much smaller reactor sizes for a given amount of IMPA removal than would be the case.

The extensive use of biodegradation in wastewater treatment systems is a testament to its low cost, reliable operation, and demonstrated ability to produce high quality effluents meeting stringent discharge criteria. However, not all xenobiotic compounds are biodegradable and even if biodegradation can be accomplished in the laboratory, it is necessary to demonstrate stable bioreactor operation over a period of time to use the process for waste disposal. This generally necessitates the establishment of a microbial consortium, which consistently degrades the waste material to suitable levels and can be reliably settled or otherwise recycled within the treatment system.

An SBR was selected for the sarin biodegradation study because of the relative kinetic and operational advantages this reactor configuration offers for the treatment of chemical wastes.[10]

Efficiency - SBRs model as a continuous flow stirred tank reactor followed by a plug flow reactor, the ideal volumetric configuration for unsteady state activated sludge systems.

Batch Operation - SBRs provide a kinetic advantage over continuous flow systems and are therefore smaller for the same degree of treatment. They also permit batch analysis and toxicity testing prior to discharge; an important feature for the treatment of chemical wastes.

Robust nature - SBRs are intentionally operated over a range of substrate concentrations, pH conditions, and oxygen concentrations allowing selection of a diverse and robust population of microorganisms.

There are four basic cycles of the SBR - Fill (feed addition; aeration and agitation yielding oxidation and mineralization of organics), React (aeration and agitation yielding oxidation and mineralization of organics), Settle (concentration of biomass at the bottom of the reactor) and Draw (removal of clarified, biodegraded effluent) wastes.[10] These cycles are repeated in a single tank on a periodic basis.

2. MATERIALS AND METHODS

2.1 Starting Materials.

Activated sludge was obtained from the Back River Wastewater Treatment plant in Essex, MD. The GB was munitions-grade material stabilized with tributylamine. Hydrolysis was conducted at room temperature with 7.18 wt % GB added gradually to aqueous NaOH (3 mol NaOH per mole GB). Gas chromatography/mass spectrometry (GC/MS – ACT Method 032, Cat. No. 03201) analysis of a chloroform extract of the hydrolysate (1 mL of chloroform, 50 mL hydrolysate) showed no GB present with a method detection limit of 0.02 μg/mL.

2.2 Equipment.

A New Brunswick BioFlo 3000 fermentation unit was used as an SBR. A timer operating a normally closed valve attached to an inhouse air supply controlled air sparging. Agitation was provided by motor driven impellers, controlled by a timer. The pH of the reactors was initially controlled by the bioreactor unit's acid feed, which was attached to a supply of 10% HCl. Subsequent pH control was achieved by adjusting the pH of the caustic feed solution to approximately 7.5 prior to feeding.

2.3 MICROTOX® Assays.

The MICROTOX® Bioassay exposes a bioluminescent marine bacterium (*Vibrio fischeri*), to a sample of unknown toxicity and measures the change in light

output as the means of determining effects on the organisms. The reduction in light output is a direct indication of metabolic inhibition. The bacterium was cultured by Azur Environmental (Carlsbad, CA) and shipped in lyophilized form. The bacterium (stored frozen) was re-hydrated immediately before testing. Each bioassay used less than 3 mL of sample and was performed in a temperature-controlled photometer. Samples were diluted with MICROTOX® diluent, and pH and salinity adjustments were conducted as needed. The assays were performed in glass cuvettes in temperature-controlled wells of a photometer. For optimum accuracy in predicting toxicity the assay must have a minimum of four dilutions exhibiting a dose response. At 5 and 15 min, the control and treatment groups were measured for light output. Data were analyzed using the MICROTOX® test protocol software to determine the EC_{50} (the effective concentration causing a 50% reduction in light output).

2.4 *Daphnia magna* Assays.

Daphnia magna are commonly used freshwater crustacean test organisms that constantly filter feed on particulate matter suspended in the water column. Their rate of filtration is 2.8 mL/hr, which greatly sensitizes them to toxic conditions in the water.[11]

Test organisms were originally obtained from Dr. Freida Taub, University of Washington (Seattle, WA), and cultured using techniques described by Goulden et al.[12] Culture/dilution media was supplied from well water that was passed through a treatment system containing a micronizer (air injection), limestone pH adjustment, Zeata Sol iron removal system, carbon filtration, and UV sterilization. *Daphnia magna* were fed a mixture of vitamin-enriched algae, *Selenastrium capricornutum*, *Ankistrodesmus falcatus*, and *Chlamydomonas reinhardtii* obtained from the University of Texas Culture Collection.* The effluent was diluted in two replicates producing the following concentrations 50.0, 42.5, 35.0, 27.5, 23.0, 20.0, and 17% vol/vol. *Daphnia* reared from third generation post-acclimated adults were used in testing. Neonates (< 24-hr old) were placed in 250 mL glass beakers containing 100 mL of sample. Beakers were placed into a temperature-controlled room at 20 °C, with a light dark cycle of 16:8. All testing conformed to Environmental Protection Agency (EPA) standard guidelines.[13] At 24 and 48 hr, the organisms were checked for immobilization by gently touching the organisms with a Pasteur pipette. If they could not swim actively for 15 sec, they were considered immobilized. The EC_{50} (effective concentration that immobilizes 50s% of the organisms) calculations were performed using the Probit Analysis contained in the Minitab statistical software package.**

*Personal communication with R. O'Neil, University of Texas, Austin, TX.
**Minitab, Incorporated, State College, PA.

2.5 Ion Chromatography Analysis of IMPA and MPA.

Water used in this analysis was distilled and deionized (10-14 meg/cm) using a Barnstead ACS Megapure System (Barnstead/Sybron, Boston, MA). Analytical grade sodium bicarbonate and sodium carbonate were obtained from Fisher Scientific Company (Fair Lawn, NJ). The ion chromatographic analysis was carried out using a Waters Millennium 2010 Data Work Station equipped with a Rheodyne injector, 2-Model 510 pumps, and a Waters Model 432 Conductivity detector (Waters Corporation, Milford, MA) with an ERIS 1000HP Autosuppressor (Alltech Corporation, Deerfield, IL). Samples were injected through a 0.22 μm syringe filter into the chromatograph. Ion chromatographic separations were performed under the following conditions: column, Dionex Ion-Pac AS14; eluent, 3.5 mM sodium carbonate/1.0 mM sodium bicarbonate buffer; flow rate, 1.2 mL/min; injection volume, 20 μL, and detection, conductivity (10 μS sensitivity).

3. RESULTS AND DISCUSSION

3.1 Bioreactor Operation.

The critical parameters of the bioreactor operation are shown in Figure 1.

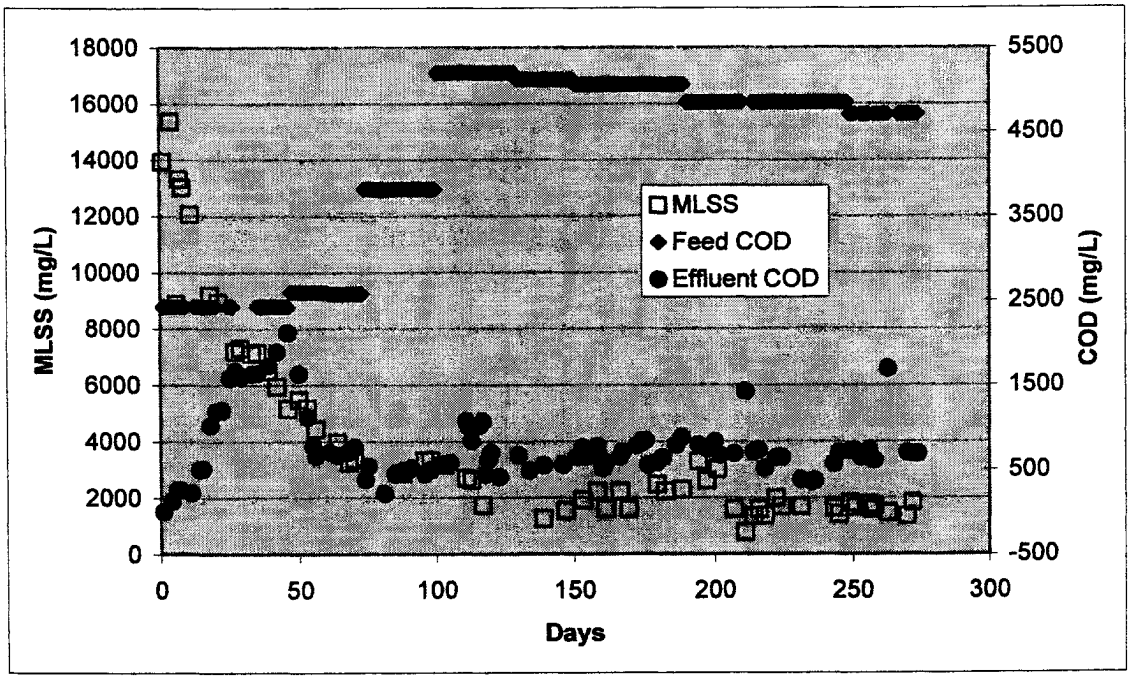


Figure 1. Feed and Effluent Chemical Oxygen Demand (COD) and MLSS of the GB Bioreactor Over an Operational Period of 274 Days.

The bioreactor was operated as a sequencing batch reactor with an 8-hr Fill cycle, a 15.25-hr React cycle, a 0.5-hr Settle cycle and a 0.25-hr Draw cycle. Initial seeding was with activated sludge that was retrieved from the Back River Wastewater Treatment plant in Essex, MD. Sludge with a mixed liquor suspended solids (MLSS) concentration of approximately 2500 mg/L was settled and the supernatant was decanted to yield an MLSS of approximately 15,000 mg/L, which comprised the bioreactor seed. No other organisms were added to the reactor.

The bioreactor was initially fed 0.25% GB hydrolysate at 1/15 of its total volume on an intermittent schedule. Feeding was occasionally discontinued to ascertain whether or not biodegradation had yet begun (as measured by COD removal). The acclimation period required approximately 46 days, during which time the effluent COD continued to rise and approach the feed COD. During this period, the reactor was fed on 33 different days, for an overall hydraulic residence time (HRT) of 20.9 days. Following day 46, the effluent COD declined precipitously for 10 days, while feedings continued on a regular 15-day HRT. At this point it was clear that the initial acclimation period had effectively ended and biodegradation had begun in earnest. Over the course of the next 8 days, the 15-day HRT feedings of 0.25% feed were continued daily, with steady or declining effluent COD measurements. At day 74, the feed concentration was increased to 0.35% and the 15-day HRT feedings of 0.35% feed continued until day 101. During this period of 0.35% feedings, COD removal averaged 88.2%. At day 101, the feed concentration was increased to 0.5%, which caused an initial rise in effluent COD. However, feeding was continued uninterrupted on a 15-day HRT, and the effluent COD declined to its previous level by day 118. From day 118 until day 274, 0.5% feeding was continued on a 15-day HRT. Occasionally the effluent COD increased, in which case feeding was interrupted to allow the effluent COD to return to lower levels. Over the course of this final 156-day period, the overall COD removal efficiency averaged 86.2%. Feed rate was generally on a 15-day HRT; although feed was occasionally stopped to correct for effluent COD excursions. During this final period, the reactor was fed on a total of 149 out of 156 days for an overall HRT of 15.7 days.

The MLSS at day 117 was approximately 1700 mg/L and on day 272 it was approximately 1800 mg/L. The average value during this 156-day period was also approximately 1800 mg/L. Thus, the reactor demonstrated a net retention of biomass during the final 156-day operational period.

3.2 Bioreactor Capacity.

The bioreactor described above was fed a maximum feed concentration of 0.5% hydrolyzed GB at a minimum HRT of 15 days. Experiments with reactors operated at other feed concentrations and/or HRTs had suggested that the maximum loading would approximate these values. A COD time course experiment conducted in a parallel reactor supports that general conclusion. The reactor had been fed 0.75% hydrolyzed sarin at varying HRTs. It was not fed for one week prior to the time course

experiment to ensure a minimal baseline COD value. Figure 2 shows the COD values in the reactor over the course of 48 hr subsequent to a single feeding with 0.75% feed at a 10-day HRT. The COD of the reactor did not return to its baseline value for approximately 30 hr, indicating that 0.75% feed at 10 days HRT was above the maximum loading. Results are consistent with a maximum attainable feed concentration of 0.5% at a 15-day HRT.

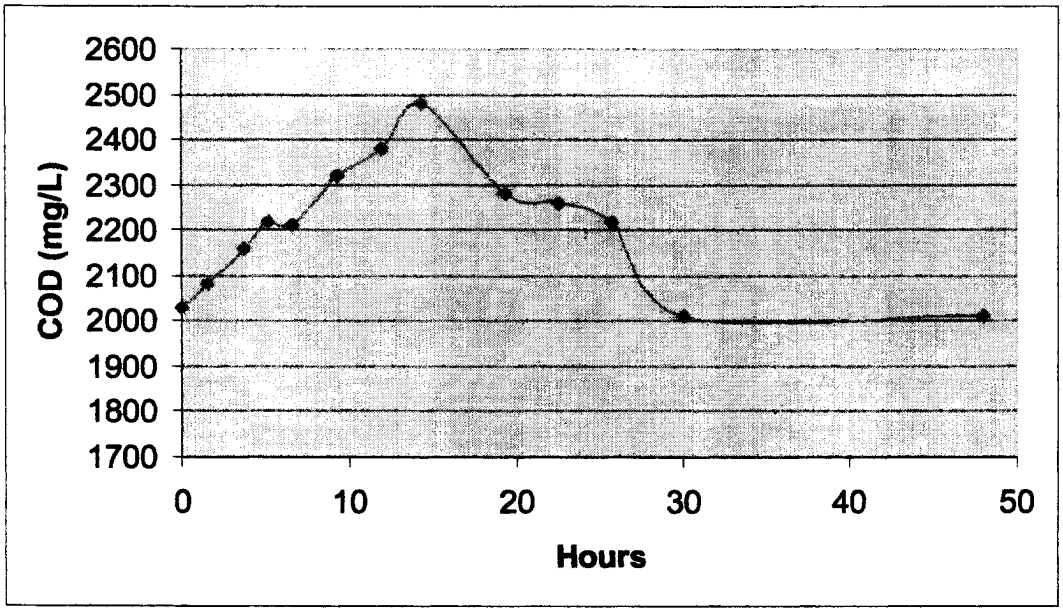


Figure 2. Time Course (48 hr) of COD Removal at 0.75% Loading and 10 Days HRT.

3.3. Aquatic Toxicity.

The bioreactor feed and effluent were analyzed for aquatic toxicity using the bacterial-based MICROTOX® test. Results (Table 1) are expressed as the effective concentration (% volume/volume) of the toxicant that produces noticeable effects on 50% of the test organisms (EC_{50}). Numerical values approaching 100% are less toxic than numerical values approaching 0%. Values are presented for 5-min and 15-min exposures of the toxicant to the test bacteria.

Table 1. MICROTOX® EC_{50} Aquatic Toxicity Values for Feed and Effluent

Sample	5 min	15 min
Feed	95.5 % vol/vol	80.8 % vol/vol
Effluent	>100 % vol/vol	>100 % vol/vol
Quality Assurance Test (phenol)	22.4 mg/L (acceptable range is 13-26 mg/L)	
Methanol (Reference material)	5.6 % vol/vol[14]	
Acetone (Reference material	2.3 % vol/vol[15]	

The bioreactor effluent toxicity was also tested on *Daphnia magna*, as described previously in Section 2 of this report. O'Bryan and Ross have developed the Chemical Scoring System for Hazardous and Exposure Identification. The scoring criteria for acute aquatic toxicity are based on a ranking from 0 to 9, with 0 being the least toxic. The 0 ranking assumes an EC_{50} >1000 mg/L for the material in question. Acetone reference standards for example, have an EC_{50} of 1.2%, corresponding to 16,590 mg/L. With a 48-hr EC_{50} of 19.8% (Table 2), the hydrolyzed sarin bioreactor effluent would correspond to 198,000 mg/L, which is well within the non-toxic range, as established by O'Bryan and Ross).

Table 2. *Daphnia magna* EC_{50} Aquatic Toxicity Values for Effluent

Sample	24 hr	48 hr
Effluent	21.3 % vol/vol	19.8 % vol/vol
Methanol (Reference material)		3.0 % vol/vol*
Acetone (Reference material		1.2 % vol/vol[14]

3.4 Quantitation of Phosphonates in Feed and Effluent.

The IMPA and MPA in the feed and in the effluent were quantified using ion chromatography. Results (Table 3) show removal of IMPA to undetectable levels (<0.07 mM, or >99.6% removal efficiency).

Table 3. Ion Chromatographic Quantitation of IMPA and MPA in the Bioreactor Feed and Effluent

Analyte	FW	Feed (mg/L)	mM	Effluent	mM	Detection Limit
IMPA	138.10	2821	20.43	Non-detect	0	0.07 mM
MPA	96.02	250.4	2.60	2563	26.69	0.05 mM
Total			23.03		26.69	

3.5. Removal of MPA from Effluent by Phoslock™ Binding.

Hydrolysis followed by bioremediation effectively degrades sarin by converting it into nontoxic MPA. However, large quantities of MPA should not be discharged into open waters because of its high phosphorus content, which can cause eutriphication. Biological phosphorus removal is possible[16] but requires large amounts of carbon to be added to the reactor. Another possible approach is removal by chemical binding to a solid material. We found that MPA, as its sodium or potassium

*Minitab, Incorporated, State College, PA.

salt, is very effectively absorbed by Phoslock™. This modified clay was developed for the improvement of fresh water lakes and rivers that had been contaminated by inorganic phosphate, largely from fertilizer run-off, and it irreversibly absorbs dianionic inorganic phosphate.

We found that Phoslock™ has a high affinity for dianionic MPA and tested it on the solution from degraded sarin. A 2 mL solution, 0.032M in dianionic MPA was treated with varying amounts of a Phoslock™ slurry in water, which is approximately 50 wt % solids. The mixtures were left for 1 hr, filtered, and the fraction of MPA in the filtrate was monitored as a function of the weight of Phoslock™ per milliliter of solution by ^{1}H NMR spectroscopy (Figure 3). The absence of the signal of the methyl group of MPA in the filtrate verified total uptake with the appropriate amount of slurry. The 500 MHz NMR spectrum was obtained on a Varian INOVA spectrometer with suppression of the signal of ^1H_2O and external TSP in D_2O as reference and deuterium lock, and in the limit, we saw no CH_3 signal of MPA, which is a doublet at 1.07 ppm, J_{P-H} = 16 Hz. We have not fully tested the limits of detection of MPA by the ^{1}H NMR spectrum, but on the basis of tests with authentic MPA we can reach a detection limit of 10 ppm within a reasonable acquisition time and recent observations under conditions similar to those described are showing that uptake of MPA is complete within a few minutes.

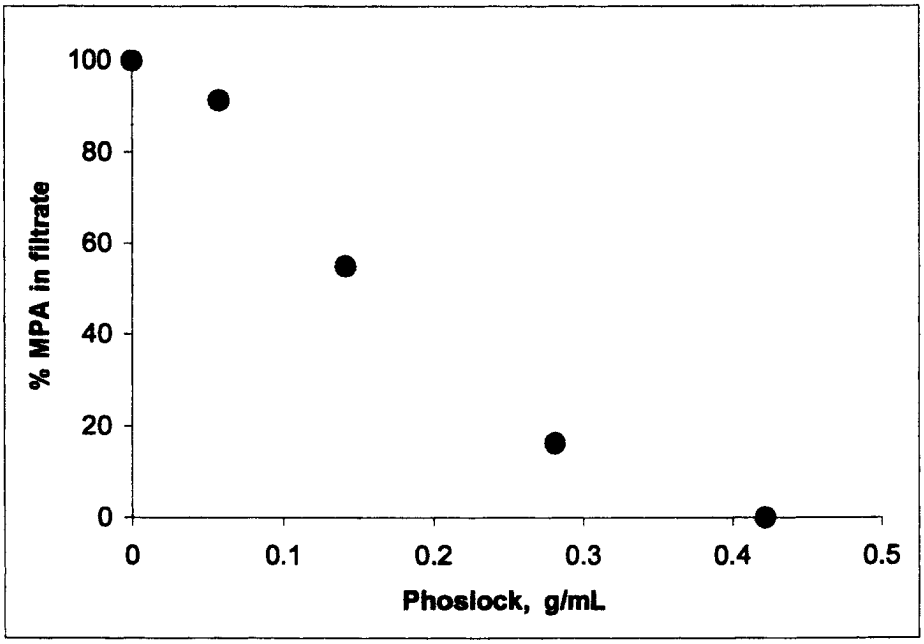


Figure 3. % MPA in Effluent as a Function of Phoslock™ Concentration.

4. CONCLUSIONS

The chemical oxygen demand (COD) and mixed liquor suspended solids bioreactor operational data indicate that a reactor seeded with a high concentration of

activated sludge can acclimate to the biodegradation of hydrolyzed sarin as its sole carbon source in approximately 46 days. Feed concentrations were gradually increased to 0.5%, and the reactor was operated for 156 days at an overall HRT of 15.7 days. These conditions facilitated COD removal with an overall efficiency of 86.2% during the 156-day stable operational period. Biomass concentration initially decreased as the reactor acclimated to the feed, then subsequently equilibrated at approximately 1800 mg/L.

Efforts made in other similar reactors to increase the loading inevitably resulted in an overload condition, and the time course of COD removal described above suggested that this reactor was operated at close to the maximum capacity. Therefore, the COD removal rate and the feed concentration may be approximately optimized in these conditions, at least with respect to the particular biomass with which this reactor was seeded.

MICROTOX® analysis showed the feed and effluent were essentially non-toxic, although the effluent was further detoxified as compared to the feed. *Daphnia magna* testing of the effluent also showed a very low level of toxicity.

Ion chromatographic analysis of *o*-isopropyl methylphosphonic acid (IMPA) and methylphosphonic acid (MPA) levels in the feed and effluent were consistent with a quantitative conversion of IMPA to MPA. The IMPA was undetectable in the effluent with a detection limit of 0.07 mM. Other IMPA-related phosphonates undetected in the analysis of the feed may similarly have been converted to MPA, thereby accounting for the overall increase in molarity of phosphonates in the effluent versus the feed.

The residual MPA from the effluent was effectively encapsulated in Phoslock™, thereby providing a high quality effluent of low toxicity and low phosphorus content.

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